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Enhancement of Gene Therapy Specificity for Diffuse Colon Carcinoma Liver Metastases with Recombinant Herpes Simplex Virus [Scientific Paper]

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Abstract TOP

Objective: The authors determined whether a recombinant herpes simplex virus (HSV) vector could destroy human colon carcinoma cells *in vitro* and whether the vector would selectively replicate in colon carcinoma liver metastases but not surrounding hepatocytes *in vivo*.

Background: The HSV vector hrR3 is defective in the gene encoding ribonucleotide reductase and contains the lacZ reporter gene. Ribonucleotide reductase is expressed in actively dividing cells and generates deoxyribonucleotides for DNA synthesis. hrR3 replicates only in actively dividing cells that can provide ribonucleotide reductase in complementation, but not in quiescent cells such as normal hepatocytes.

Methods: hrR3- mediated lysis of HT29 human colon carcinoma cells was first determined *in vitro*. For *in vivo* studies, athymic BALB/c nude mice underwent intrasplenic injection of HT29 and intrasplenic injection of hrR3 7 days later, and were killed 7 days after viral injection. Their livers were examined histochemically for lacZ expression.

Results: All the HT29 cells were destroyed *in vitro* when hrR3 was added at a titer of 1 plaque-forming unit per 10 tumor cells. One hundred one of 105 tumor nodules examined in liver sections from mice treated by intrasplenic injection of hrR3 demonstrated lacZ expression. Minimal betagalactosidase activity was present in normal liver.

Conclusions: The hrR3 HSV vector effectively destroys HT29 human colon carcinoma cells at very low multiplicities of infection. Differential expression of ribonucleotide reductase between liver metastases and normal liver allows hrR3 to selectively replicate in tumor cells with minimal replication in surrounding normal liver. Further investigation of HSV-based vectors as oncolytic agents for liver metastases is warranted.

Article Outline

Figures/Tables

This year, colorectal carcinoma will develop in approximately 135,000 people in the United States. Although mortality rates from this disease are declining, more than 55,000 people are expected to die of colorectal carcinoma in 1996.1 Liver metastases remain the most prevalent form of metastatic disease, and it is estimated that liver metastases will develop in 75% of patients who die of colorectal carcinoma.2 Although a small proportion of patients with colorectal carcinoma liver metastases may benefit from surgical resection, most patients have multiple metastases and are not candidates for surgical resection. Patients with multiple metastases have been treated with a variety of modalities, including intravenous chemotherapy, regional chemotherapy, cryosurgery, and chemoembolization. However, these approaches remain palliative; rarely-if ever-are patients with diffuse metastases cured by these treatments.2

Several gene therapy strategies have been examined for their therapeutic potential in the treatment of cancer. These approaches have been based on 1) introduction of genes that produce multiple copies of RNA decoys; 2) expression of transdominant proteins that can functionally replace mutant or deleted cellular proteins; 3) modification of adoptively transferred T cells; 4) expression of cell surface antigens to enhance the antitumor response; 5) expression of ribozymes that cleave specific DNA sequences; 6) intracellular production of antibodies to bind to specific proteins; 7) expression of foreign enzymes that render cells susceptible to otherwise nontoxic prodrugs; 8) production of intracellular toxins that lead to cell death; 9) modification of hematopoietic stem cells to decrease toxicity from chemotherapy, or 10) infection with oncolytic viruses that can themselves destroy tumor cells.3

Numerous vehicles for delivery of genes to both normal cells and cancer cells have been developed, including retrovirus, adenovirus, vaccinia virus, adeno-associated virus, and herpes virus.4 Most of the previously described gene therapy approaches for cancer require direct intratumoral injection of these vectors.5-7 This strategy is not feasible for patients with *diffuse* liver metastases. Hurford and colleaguesa treated diffuse hepatic micrometastases in mice, created by splenic injection of sarcoma and breast carcinoma cell lines, with intrasplenic injection of a retroviral producer cell line. They demonstrated selective gene transfer to tumor deposits. There are several drawbacks to retroviral vectors, including the theoretical capability of causing neoplastic transformation of normal cells by insertional mutagenesis. Accordingly, we have investigated a strategy using herpes simplex virus (HSV) type 1 vectors for treatment of *diffuse* liver metastases.

Herpes simplex virus has been explored as a vehicle for gene transfer into the central nervous system, but studies using HSV have demonstrated cytotoxicity from cellular lysis.**9,10** These apparently negative characteristics of HSV vectors can be adapted for therapeutic purposes in the treatment of cancer. Entry of wild-type HSV into cells leads to a sequential cascade of viral gene expression that ultimately results in the production of multiple progeny virions and cell death.11 Herpes simplex virus vectors demonstrate significant oncolytic activity and reporter gene transfer in experimental brain tumor models.12

The normal liver is similar to the brain because it has minimal mitotic activity.13 In contrast, liver metastases demonstrate significantly greater mitotic activity. The activity of enzymes necessary for DNA replication, such as ribonucleotide reductase, is increased in tumors compared with normal tissues.14 Accordingly, one strategy to develop HSV vectors that selectively lyse diffuse liver metastases rather than normal hepatic parenchymal cells involves deletion of specific genes necessary for viral replication, such as ribonucleotide reductase. Such vectors would only be able to replicate in actively dividing tissue that could provide ribonucleotide reductase in complementation.15,16

In the current study, we have assessed the feasibility of employing a mutant HSV vector in the treatment of diffuse colon carcinoma liver metastases. We have examined paired patient specimens representing normal liver and colon carcinoma liver metastases for ribonucleotide reductase expression, and confirmed that expression is virtually undetectable in normal liver. In contrast, extremely high levels of ribonucleotide reductase were found in colon carcinoma metastases. Then we examined the ability of the ribonucleotide reductase deficient HSV vector (hrR3) to infect and destroy HT29 human colon carcinoma cells *in vitro*. hrR3 destroyed HT29 cells at titers of only 1 plaque-forming unit per 10 tumor cells. Finally, we injected hrR3 intrasplenically into nude mice bearing diffuse HT29 liver metastases. hrR3 specifically and efficiently targeted diffuse liver metastases. Negligible hrR3 infection of normal liver was identified.

MATERIALS AND METHODS TOP

Cell Lines, Tumor Specimens, Antibodies, and Viral Vectors TOP

The human colon carcinoma cell line HT29 was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium with Hamm's F12 supplement and 8% (v/v) fetal calf serum. Human tissue specimens were immediately frozen in liquid nitrogen in the operating room and stored until further processing.

The monoclonal antibody MAS 378 AD203 (Accurate Chemical and Scientific Corp., Westbury, NY) recognizes the M1 subunit of ribonucleotide reductase. The monoclonal antibody A-5441 (Sigma Chemical Co., St. Louis, MO) recognizes beta-actin.

The hrR3 HSV vector was obtained from S. Weller (Connecticut Medical School, University of Connecticut Health Center, Farmington, CT) and is defective in ribonucleotide reductase expression.15,16 This vector contains the *Escherichia coli* lacZ gene inserted into the ribonucleotide reductase gene locus. The lacZ gene is driven by the ICP6 immediate-early gene promoter. hrR3 was passaged on parental African Green Monkey (Vero) cells and stored in stocks at -80 C before use.

Western Blot TOP

For Western blot analysis, tumor tissue was homogenized in 50 mmol/L Tris, pH = 8; 150 mmol/L sodium chloride; 0.2% sodium azide, 100 µg/mL phenylmethylsulfonyl fluoride; 1 mg/mL aprotinin; and 1% Triton X-100. Total protein concentration was measured using the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL). Lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, and transferred to nitrocellulose filters by electroblotting at 4 C. After blocking for 1 hour in phosphate-buffered saline (PBS) containing 5% dry milk, the filters were incubated with MAS 378; washed in PBS containing 1% dry milk and 0.2% Tween-20; incubated with horseradish peroxidase-conjugated antimouse antibody (Amersham Corp., Arlington Heights, IL); washed in 150 mmol/L sodium chloride, 10 mmol/L Tris, pH = 8, 0.05% Tween-20; and specific proteins were detected using an enhanced chemiluminescence system (Amersham Corp.). Filters were reprobed with A-5441 to detect beta-actin.

In Vitro Cell Infection Assay TOP

HT29 cells were plated in 96 well plates at a concentration of 5000 cells per well and allowed to grow for 48 hours. The media were removed and the cells were washed with serum-free media. Then hrR3 virus in 50 µL of serum-free media was added to the cells in multiplicities of infection (number of plaque-forming units per cell) ranging from 0.0001 to 1. The cells were gently shaken every 15 minutes for 45 minutes at 37 C, and 50 µL Dulbecco's modified Eagle's medium-F12 supplemented with penicillin, streptomycin, and 16% fetal calf serum was then added after this initial viral adsorption period. Six days later, the media were replaced with RPMI 1640 without phenol red containing 0.5 mg/mL thiazolyl blue (MTT; Sigma Chemical Co.) for 1 hour at 37 C.[17] The media were removed, and formazan crystals were solubilized with 50 µL dimethyl sulfoxide (DMSO). After the plate was vigorously shaken, the optical density of each well was measured using an automatic plate reader (Anthos HT2, Anthos Labtec Instruments, Salzburg, Austria) with a 550-nm measurement wavelength and a 650-nm reference wavelength. The percentage cell survival was determined by calculating the ratio of $OD_{550/650}$ of hrR3-infected cells to the $OD_{550/650}$ of mock infected cells. All experiments were performed in quadruplicate.

Hepatic Metastasis Model and Treatment with Herpes Virus Vector TOP

Animal studies were performed in accordance with guidelines issued by the Massachusetts General Hospital Subcommittee on Research Animal Care. Pathogen-free, 4- to 5-week old male athymic BALB/c nude mice were allowed to acclimate for 1 week. Cells were detached from plates in 5 mmol/L edetic acid in PBS and resuspended in Hank's balanced salt solution, free of calcium and magnesium; 5×10^6 cells in a single-cell suspension were injected intrasplenically, as described.[18] One week later, either 1×10^7 plaque-forming units of hrR3 in 100 µL of PBS or 100 µL of PBS alone were injected into the spleen. One week later, the mice were killed and their livers were examined for lacZ expression.

Liver Sections and Histochemical Staining TOP

Livers were snap frozen in liquid nitrogen, and frozen sections were prepared. Sections were fixed in 4% paraformaldehyde in 10 mmol/L sodium phosphate and 0.9% sodium chloride (pH = 7.3) and then washed with PBS. Slides were incubated for 48 hours at 37 C in a solution containing 35 mmol/L potassium ferricyanide, 35 mmol/L potassium ferrocyanide, 2 mmol/L magnesium chloride, 0.01% sodium deoxycholate, 0.02% Nonidet P40, and 0.2% of a solution containing 40 mg 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal; Sigma Chemical Co.) in 1 mL of dimethylformamide at pH = 7.3. The slides were rinsed with water and counterstained with cresyl violet.

RESULTS TOP

Ribonucleotide Reductase Expression in Human Colon Carcinoma Metastases and Normal Liver TOP

We examined ribonucleotide reductase expression in biopsies of colon carcinoma liver metastases and adjacent normal liver from patients by Western blot analysis. As expected, ribonucleotide reductase expression in the relatively quiescent liver was extremely low compared with the high levels detected in the colon carcinoma metastases (**Fig. 1**). These data supported the feasibility of selectively targeting liver metastases with an engineered herpes viral vector defective in ribonucleotide reductase that should replicate only liver metastases where ribonucleotide reductase is available in complementation.

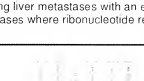

Figure 1. Expression of ribonucleotide reductase M1 subunit (RR) in paired specimens representing human colon carcinoma liver metastases (liver met) and adjacent normal liver (nl liver) from three patients measured by Western blot analysis. Equivalent amounts of protein were loaded in each lane as demonstrated by beta actin expression.

Herpes Simplex Virus Vector-Mediated Lysis of HT29 Colon Carcinoma Cells *In Vitro* TOP

On the basis of the aforementioned findings, we sought to examine the ability of a ribonucleotide reductase defective HSV vector to infect and destroy human colon carcinoma cells *in vitro*. The hrR3 HSV vector is defective in the large subunit of the viral ribonucleotide reductase because of an insertional mutation. This vector has the *E. coli* lacZ gene driven by the ICP6 immediate early promoter inserted into the ribonucleotide reductase gene locus. We wanted to examine the ability of this HSV vector to destroy tumor cells in culture, regardless of the pH and nutrient changes associated with prolonged incubation. Accordingly, we chose 6 days as an

endpoint for the assay, and first determined conditions that would allow log phase growth of the HT29 cells for 6 days after initial seeding.

Six days after infecting HT29 human colon carcinoma cells with hrR3 at multiplicity of infection (MOI) values ranging from 0.0001 to 1.0, we quantitated the number of viable cells using the colorimetric MTT assay. We also quantitated the number of viable uninfected control cells. hrR3 destroyed approximately 70% of the HT29 cells infected at a titer of 1 plaque-forming unit per 100 tumor cells (multiplicity of infection = 0.01; Fig. 2). Virtually 100% of the cells were destroyed after 6 days when hrR3 was added a titer of 1 viral particle per 10 tumor cells (multiplicity of infection = 0.1). Cytopathic effects were observed in culture as early as 24 hours after infection. These data indicate that hrR3 is an extremely effective cytopathic agent against HT29 cells *in vitro*.

Figure 2. hrR3-mediated lysis of HT29 colon carcinoma cells *in vitro*. The percentage of cells destroyed 6 days after infection with hrR3 was quantitated by the MTT assay. Data are presented as the mean ± standard deviation of quadruplicate experiments.

We also examined the ability of the ICP6 promoter to drive expression of the lacZ gene in the HT29 cells. HT29 cells were fixed in glutaraldehyde 72 hours after hrR3 infection and stained with X-gal. Strong lacZ expression was detected (data not shown), indicating that we would be able to identify hrR3 infected cells *in vivo* by staining for lacZ expression.

Splenic Injection of Herpes Virus Vector to Infect Liver Metastases TOP

On the basis of having demonstrated differential expression of ribonucleotide reductase in normal liver compared with metastases and highly effective hrR3-mediated destruction of colon carcinoma cells *in vitro*, we next sought to determine if hrR3 would selectively replicate in diffuse liver metastases *in vivo* when injected into the portal vein. To produce diffuse liver metastases, 5×10^6 HT29 cells were injected into the spleens of nine nude mice. Seven days later, 1×10^7 plaque-forming units of hrR3 were injected intrasplenically. The animals were killed 7 days later, and liver sections were examined histochemically for lacZ gene expression. One hundred five tumor nodules were examined in several liver sections from all nine mice, and lacZ gene expression was detected in 101 (96%) of these nodules. The extent and distribution of lacZ gene expression were relatively similar for all the tumor nodules (**Fig. 3**). Five to 25% of HT29 colon carcinoma cells stained positive for lacZ expression in most of the tumor nodules. We also detected a minimal amount of beta-galactosidase activity in areas of normal liver that had no clear evidence of tumor metastases. In a separate set of experiments, mice bearing liver metastases introduced via the spleen were injected with PBS only (no hrR3 virus). Tumor nodules examined in several liver sections demonstrated no evidence of lacZ expression. No beta-galactosidase activity was noted in normal liver in these animals.

Figure 3. Histochemical detection of lacZ expression in HT29 liver metastases 7 days after splenic injection of hrR3. (A) Tumor nodule examined at 100× magnification. (B) Tumor nodule examined at 200× magnification. Sections were counterstained with cresyl violet.

DISCUSSION TOP

Our initial interest in HSV vectors for gene delivery and destruction of liver metastases arose from experimental results using HSV vectors to treat brain tumors.[19,20] The ribonucleotide reductase deficient hrR3 vector can mediate complete tumor regression and 20% long-term survival in rats harboring an intracerebral 9L neoplasm. Furthermore, negligible hrR3 infection of surrounding normal astrocytes and endothelial cells occurs. This therapeutically favorable differential susceptibility of malignant *versus* normal brain tissue to cytolytic infection by hrR3 presumably results from the extremely high levels of mitotic activity within tumors compared with the surrounding normal brain tissue.[12]

We have examined the feasibility of this strategy to treat liver metastases. We assumed that ribonucleotide reductase levels in normal liver would be much lower than in colorectal carcinoma liver metastases. We were unable to locate any reports describing ribonucleotide reductase levels in colorectal carcinoma. Accordingly, we examined several pairs of normal liver and liver metastases specimens by Western blot analysis and confirmed our assumption. Although the difference in expression was quite dramatic, very low levels of ribonucleotide reductase expression were detected in normal liver. Further analysis by immunohistochemistry is necessary to identify the source of this expression.

Several features of hrR3 render it an advantageous vector for tumor gene therapy. First, hrR3 is replication conditional and will multiply in dividing cells, such as metastases, and not multiply in nondividing cells, such as hepatocytes.[19] This relative selectivity results from tumor upregulation of ribonucleotide reductase, thereby allowing complementation for hrR3 replication. Second, in addition to its inherent cytopathic effect, hrR3 may be used to deliver cytotoxic genes to tumor cells. For example, hrR3 possesses an

endogenous thymidine kinase gene that confers chemosensitivity to ganciclovir.12 The HSV genome also can accommodate exogenous genes. It is estimated that up to 30 kb of the HSV genome can be replaced by exogenous sequences.21 Third, hrR3 provides these functions at very low multiplicities of infection.

Direct intratumoral inoculation of hrR3, as was performed for treatment of experimental brain tumors, is not a feasible strategy to treat diffuse liver metastases. Accordingly, we examined the efficacy of intraportal delivery of hrR3 via splenic injection. The efficacy and specificity of lacZ gene expression in tumor cells were striking. Ninety-six percent of histochemically examined tumor nodules stained positive for lacZ expression. The ratio of lacZ expression in tumor nodules was extremely high compared with normal hepatic parenchyma. However, some beta-galactosidase activity was detected in liver sections in which no HT29 tumor cells could be identified by routine cresyl violet counterstaining. There are several potential explanations for this finding. Low levels of ribonucleotide reductase expression in endothelial cells and hepatocytes may have been sufficient to allow complementation for hrR3 replication. Alteratively, insufficient levels of ribonucleotide reductase in these cells may have resulted in aborted replication after lacZ gene expression.19 Some of the positive staining in normal liver also could be a result of hrR3-infected isolated HT29 cells scattered throughout the liver. It is unlikely that much of the staining in normal liver was due to endogenous beta-galactosidase activity. We carefully controlled the pH of the staining solutions to suppress endogenous beta-galactosidase activity; endogenous mammalian beta-galactosidase is active only in acidic conditions.22 Additional studies are necessary to examine each of these possible explanations for the low level staining present in normal liver.

hrR3 gene expression, as assessed by lacZ expression, was confined to no more than 25% of tumor cells within any tumor nodule. Most often, 5% to 10% of the tumor cells expressed lacZ. LacZ expression was assessed only at 7 days postinfection. Further studies are warranted to assess the level of lacZ expression at various time intervals postinfection to gain insight into the percentage of tumor that ultimately becomes infected with hrR3.

It is unlikely that infection of every tumor cell in a liver could be achieved by portal injection of hrR3, and a few surviving cells may be sufficient to re-establish a tumor. However, this problem may be surmountable. Numerous studies demonstrate that viral gene expression is required in only a portion of a tumor for complete tumor regression.23 The death of uninfected tumor cells adjacent to transduced cells has been termed the bystander effect.24 This effect may be exploited to enhance tumor destruction. As previously noted, hrR3 possesses a viral thymidine kinase gene that converts ganciclovir to a toxic metabolite. Ganciclovir treatment has been shown to potentiate the antitumor effect of hrR3 in the treatment of gliosarcomas in a rat model.12 Although the mechanisms involved in this potentiation are not elucidated fully, the bystander effect probably is an important component of the enhanced tumor regression. Assessment of the effects of ganciclovir on hrR3-infected colorectal carcinoma is necessary for further development of therapeutic strategies.

Thus, differential expression of ribonucleotide reductase between liver metastases and normal liver allows hrR3 to selectively replicate in tumor cells with minimal replication in surrounding normal liver. Intraportal injection of hrR3 results in expression of the *E. coli* lacZ gene in more than 95% of tumor nodules. These results warrant further investigation into HSV vector-based therapies against liver metastases.

Acknowledgment TOP

The authors thank Maureen Chase for her help with herpes viral production and measurement of viral titers.

Paper Discussion TOP

DR. SAMUEL A. WELLS, JR. (St. Louis, Missouri): The basic premise of this paper is that gene modification of the herpes virus, by interrupting the ribonucleotide reductase locus, causes selective infection in tumor tissue. This can only be proven by comparing *in vivo* infection of this modified virus to the relative infectivity of genetically unmodified wild-type herpes virus for normal murine liver cells and HT-29 tumor cells, and it seems that this should have been the control.

Some wild-type viruses have been shown to specifically infect and persist in tumor tissue but not normal tissue after administration. Thus, the gene modification may have no relevance to the HT-29 tumor specificity of the virus. Furthermore, viruses typically have a trophism for specific tissue and in liver cells, may not support replication of the herpes virus as well as human tumor cells. This species difference alone may explain the observed results. Data regarding this argument were not presented, and I hope that the authors will be able to clarify my confusion.

DR. WILLIAM C. WOOD (Atlanta, Georgia): I would also like to congratulate Dr. Tanabe and his two investigators on their results. This is a fascinating probe that they have. Ninety-six percent of the nodules had some lacZ expression, but only 5% to 25% of the tumor cells had evidence of infectivity with this agent, often only 5% to 10%.

My first question to Dr. Tanabe is, is that sufficient to eradicate tumor from direct cytotoxicity or from bystander cytotoxicity? What cytolysis did they find at 7 days? It did not seem striking to the untrained eye on the biopsy just shown.

My second question is, did you inject this virus into the spleens of normal mice to see if there are normal areas in the liver or elsewhere in the body that would take up this virus and be infected with herpes simplex virus (HSV)? To ask the same question in a

different way. do you have any phase 1 toxicity data on the injection of this agent in the murine system?

Third, do you have any phase 2 data from this system? Did these tumor nodules that took up lacZ and appeared to be infected with your HSV variant show a complete response rate or a partial response rate that would enable us to have any evidence as to the direct cytotoxicity of this agent?

I would congratulate you for opening an approach to viral targeting that takes advantage of this potential differential.

DR. TIMOTHY J. EBERLEIN (Boston, Massachusetts): Dr. Tanabe and his colleagues present a potentially useful strategy for the treatment of colorectal liver metastases. Although surgical excision of colorectal metastases offers a 25% to 30% long-term survival rate, the majority of these patients have recurrence. Therefore, a potentially useful treatment that would treat an entire liver with potential micrometastases is desired.

Within this background, Dr. Tanabe takes advantage of the fact the normal liver had minimal mitotic activity. In marked contrast, the tumor in the liver had very high mitotic activity and therefore would utilize the enzymes necessary for DNA replication. One of these enzymes, ribonucleotide reductase, has been shown to be increased in tumors.

The central observation in the study is that by utilizing Western blot analysis, Dr. Tanabe has shown us that normal liver specimens have virtually undetectable ribonucleotide reductase expression yet there is an extremely high level in colon carcinoma liver metastases. It is this differential that forms the basis of a potential treatment utilizing this strategy.

My first question is, what is the expression of the ribonucleotide reductase in other more actively dividing tissues such as epithelial cells in the gastrointestinal tract? If this strategy were to be combined with a liver resection and regenerated liver then ensued, what is the level of ribonucleotide reductase expression in regenerating liver?

This type of treatment strategy has been utilized, as mentioned, for experimental brain tumors. In that model, direct intratumoral inoculation is utilized. This is not feasible for the treatment of diffuse liver metastases.

Dr. Tanabe has shown the efficiency and specificity of tumor cell infection using lacZ gene expression. This is striking. However, there is some beta galactosidase activity detected in normal liver tissue yet no tumor cells are identified by routine counterstaining. Does this imply that retreatment with the herpes simplex virus (HSV) vector will increase beta galactosidase activity in normal liver, thereby making this treatment less specific and potentially more toxic?

In a related issue, although Dr. Tanabe has shown elegant uptake in tumor tissue of this HSV vector, how much *in vivo* tumor cytotoxicity is identified? Did the cytotoxicity actually correlate with gene update? And what might the effect of tumor necrosis, as is often seen in liver metastases, be on the treatment strategy?

Although 96% of the tumor nodules were identified by lacZ gene expression, is there an explanation for why the other nodules were not identified? Similarly, because only 5% to 25% of each tumor nodule stained positive, do you have an explanation for the lack of staining of the remaining tumor nodules?

Finally, I would encourage further studies, as was proposed in the discussion in the manuscript, on the herpes viral thymidine kinase strategy because this model may be an ideal model to study the bystander effect, which has been based on an *in vitro* assay and the mechanism of which has not been satisfactorily worked out in an *in vivo* model.

DR. JEFFREY A. NORTON (St. Louis, Missouri): I would like to rise to say that I enjoyed this paper very much. In our laboratory, we have done similar work with vaccinia virus. We have deleted the ribonucleotide reductase gene, the thymidine kinase gene and the hemagglutinin gene in that virus and had similar results. *i e*, relative specificity to the tumor. But I think that the word relative is important in analyzing these data. I would like to ask a few questions, many of which have been already described by the other discussants.

What transgenes do you plan to add to the herpes simplex virus?

The current strategy relies primarily on infection of almost 100% of the tumor cells. The specificity, that is the infection rate of tumor cells *versus* normal liver tissue, is not that great. Therefore, I expect that you will see some toxicity, that is infection of other rapidly dividing cells, like the bone marrow and intestinal mucosa.

Therefore, how do you plan to improve the specificity to see more infection of the tumor cells and less infection of normal tissue?

DR. KENNETH K. TANABE (Closing Discussion): I would again like to give credit to others in my research group: Drs. Carroll, Takahashi, and Chiocca.

The presence of hrR3 herpes virus in cells other than tumor cells remains a major concern for us. Liver cell populations other than hepatocytes, such as Kupffer's cells and endothelial cells, have higher replicative activity than hepatocytes. It is certainly possible that these cells account for the ribonucleotide reductase that we were able to detect in normal liver. Immunohistochemical studies are necessary to localize the source. I suspect that hrR3 can replicate in these normal cells. Furthermore, even in the absence of ribonucleotide reductase in a cell, hrR3 replication may occur, as Dr. Norton points out, albeit at significantly reduced efficiencies. Herpes can also exist in a latent state, unlike many other viruses, a state that we would not be able to detect by examination for lacZ expression. In brief, even though hrR3 is a replication conditional mutant, it is easily conceivable that it could have infected nontumor cells in the liver.

Although an intraportal injection principally targets the liver, hrR3 introduced by this route probably also makes its way into the systemic circulation. Additionally, shed virus from hrR3 that is replicating in the liver tumors may also reach the systemic circulation. Presumably, the organs at highest risk from this systemic spread are those with the highest replicative activity that can provide ribonucleotide reductase in complementation, such as hair follicles, bone marrow, and gut mucosa. As each of the discussants have pointed out, clearly we need to look for systemic hrR3 spread in our animal models. We have already embarked upon these studies with polymerase chain reaction analysis and histochemical staining, but unfortunately I do not have any data to show you today. The hrR3 construct retains an intact thymidine kinase gene which renders the virus susceptible to acyclovir or ganciclovir. We need to carefully document any systemic presence of hrR3 after intraportal injection, as well as its response to acyclovir.

There are several potential explanations for the low level of beta-galactosidase activity that we observed in normal liver. As I mentioned, some cells in normal liver expressed ribonucleotide reductase and hrR3 may have been replicating in these cells. Alternatively, some of this staining detected in what was apparently normal liver may have represented hrR3 replication in diffuse individual scattered HT29 cells that were not detected by routine staining. Some of the lacZ staining may have represented quiescent cells infected with hrR3, in which replication was aborted due to the absence of ribonucleotide reductase and lacZ expression preceded abortion of the replicative process. Lastly, mammalian cells have low levels of endogenous beta-galactosidase activity that could have accounted for some of the blue staining. However, this endogenous mammalian beta-galactosidase activity is most active at acidic pH, whereas *Escherichia coli* beta-galactosidase is most active at neutral pH. We carefully controlled the pH of the staining solutions to minimize this type of background staining. We are currently trying to address each of the possible explanations for the staining seen in normal liver.

Some of the treated tumor nodules did not have any detectable lacZ expression. All of the animals in these experiments were sacrificed at a single time point after injection of hrR3. We really do not understand the temporal sequence of events *in vivo* yet. It may be that some tumor nodules expressed lacZ at day 3 but not at day 7.

For the nodules that did express lacZ at day 7 we do not know the duration of expression. Studies designed to understand the timing of hrR3 gene expression are obviously necessary. For example, we need to know when thymidine kinase expression is maximal to determine when to introduce the pro-drug, ganciclovir.

The limited time permits me to address a couple of the other questions only in brief. We have not demonstrated *in vivo* cytotoxicity in this study. We have only demonstrated *in vitro* cytotoxicity and *in vivo* targeting. We would first like to incorporate into our animal model the use of the pro-drug ganciclovir which gets converted by thymidine kinase into a toxic metabolite.

Dr. Norton asked which transgene we plan to introduce. Herpes does carry its own thymidine kinase gene, and we will make use of it. We are also actively pursuing the introduction of cytosine deaminase as a transgene. As you can see, most of our efforts are centered around the pro-drug approach. The bystander effect seen with pro-drug strategies may yield an effective therapy with transduction of less than 100% of the tumor cells. Dr. Wells brought up an excellent point in his comments. We need to use wild-type herpes simplex virus as a control to confirm our hypothesis. Some work has been done using wild-type herpes simplex virus in the treatment of brain tumors, and wild-type herpes infects with much less specificity in that particular animal model. However, we need to examine this in our model as well.

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